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Protective Immunity in Toxoplasmosis: Correlation between Antibody Response, Brain Cyst Formation, T-Cell Activation, and Survival in Normal and B-Cell-Deficient Mice Bearing the *H-2*^{k} Haplotype

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Correlations of *Toxoplasma gondii*-specific immunoglobulin M (IgM) and IgG production, antigen-specific T-cell activation, and the number of brain cysts were compared in immunocompetent CBA/J (*H-2*^{k}), C3H/He (*H-2*^{k}), and B-cell-deficient CBA/N (*H-2*^{k}) mice. Almost all of the C3H/He mice (94%) survived in comparison to CBA/J (71%) and CBA/N (53%) mice following infection with 20 cysts of Me 49, an avirulent strain of *T. gondii*. The mortality in susceptible mice was reduced by treatment of the animals with sulfadiazine during the acute stage of infection. Decreased mortality in CBA/J and C3H/He mice as well as in B-cell-deficient mice was paralleled by formation of fewer brain cysts. The *Toxoplasma*-specific T-cell proliferation was markedly enhanced in all three strains at day 15 postinfection but not at day 45 postinfection when compared to animals not treated with the drug. In contrast, *Toxoplasma*-specific IgM and IgG levels were lower in CBA/J and CBA/N mice treated with sulfadiazine than in untreated mice of these strains. Although CBA/N mice developed almost no humoral response either with or without drug treatment, they produced fewer brain cysts than normal CBA/J mice. The results indicate a major role of cell-mediated immunity in protection against an acute *Toxoplasma* infection.

The immune response to *Toxoplasma gondii* is complex and involves humoral as well as cellular mechanisms (21). However, a major role for humoral antibody in resistance to the infection remains questionable. Gill and Prakash (12) reported that passive transfer of immune rabbit serum to rabbits and mice prior to challenge with the highly virulent RH strain of *T. gondii* did not afford protection. However, because of the extreme virulence of this strain, these results may not count against a protective role of specific anti-*Toxoplasma* antibody. Similar results were obtained by other investigators (reviewed in reference 20). On the other hand, Krahenbuhl et al. (21) found that administration of immune mouse serum to normal mice prior to challenge with graded doses of a relatively avirulent strain of *T. gondii* resulted in a significant decrease in percent mortality. Moreover, Frenkel and Taylor, using μ-suppressed mice, demonstrated that, although antibody may not be necessary to control acute infection, it may be important in controlling long-term toxoplasmosis (10). Recently, it was demonstrated that passive transfer of monoclonal antibodies which react with cell surface antigens of *T. gondii* can confer resistance against lethal challenge with this parasite (16, 25).

The barrier to passive diffusion of antibodies into the brain has been used to explain the continued proliferation of the parasite at this site while the organism is disappearing from the extraneural sites (9). However, cysts persist in tissues in which antibody is not blocked by such a barrier, e.g., heart and skeletal muscle (23).

Cell-mediated immune responses to *Toxoplasma* infection have been demonstrated as delayed hypersensitivity reactions (8, 11, 18) and as antigen-specific lymphocyte transformation (19, 29). Recently, we demonstrated that the L3T4–T-cell subset is responsible for the observed antigen-specific proliferation and that this T-cell subpopulation is regulated by a B-cell-dependent mechanism (3). In addition to the in vitro observations described above, Lindberg and Frenkel (22) showed that T-cell-defective nu/nu mice lack the capacity to develop resistance to *Toxoplasma* infection.

In the present study, we compared parasite-specific immunoglobulin G (IgG) and IgM production, antigen-specific T-cell response, and the number of cysts which developed in the brains of immunocompetent and B-cell-deficient mice bearing the *H-2*^{k} haplotype. In addition, we examined the influence of the observed humoral and cellular immune responses on cyst formation of the parasite and survival of the host.

MATERIALS AND METHODS

Mice. Swiss-Webster and C3H/He mice were obtained from Simonsen Laboratories, Gilroy, Calif., CBA/J mice were from Jackson Laboratories, Bar Harbor, Maine, and CBA/N mice were procured from the National Institutes of Health in Bethesda, Md. All mice were 6 to 8 weeks old when used.

Parasite and infection. The Me 49 strain of *T. gondii* was maintained in Swiss-Webster mice and used in all experiments. For infection, brains were removed from infected mice, triturated in a mortar, and mixed with 1 ml of normal saline. The suspension was passed through a 22-gauge needle three times, and the number of brain cysts in a 50-μl aliquot was determined microscopically. Brain suspensions were adjusted to contain 100 cysts per ml in saline and mice were infected with 20 cysts intraperitoneally (i.p.), an inoculum shown to provide a reproducible infection in preliminary experiments. In some experiments, mice were treated with sulfadiazine (a drug that converts lethal *T.*

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TABLE 1. Mortality in *T. gondii*-infected CBA/J, CBA/N, and C3H/He mice

Strain	Treatment with sulfadiazine[a]	No. of mice	Survival at 12 wk (%)
CBA/J	−	17	71
	+	14	93[b]
CBA/N	−	17	53
	+	14	92[c]
C3H/He	−	17	94
	+	14	100[d]

[a] Mice were infected with 20 cysts i.p. Fourteen of the infected mice were treated with sulfadiazine in the drinking water from days 5 to 18 p.i. The experiment was discontinued 12 weeks after infection.
[b] Values significantly different ($P = 0.119$ by Fischer's exact test) from those of CBA/J mice not treated with sulfadiazine.
[c] Values significantly different ($P = 0.018$ by Fischer's exact test) from those of CBA/N mice not treated with sulfadiazine.
[d] Values not significantly different from those of C3H/He mice not treated with sulfadiazine.

gondii infections to lifelong chronic infections) from day 5 to 18 postinfection (p.i.) to slow down parasite multiplication. The lowest dose of sulfadiazine in the drinking water (0.4 mg/ml) which protected 100% of the C3H mice was chosen to keep the infuence of the drug minimal. Survival was determined at 12 weeks p.i., because in our experience mice at this time have overcome the acute infection and developed protective immunity as evidenced by survival during the chronic state of infection.

***Toxoplasma* antigens.** *T. gondii* sonic extracts prepared as described previously (26) were used as the source of antigens.

Spleen cells. Spleen cells were prepared as described previously (3). Briefly, spleens from mice were homogenized and the erythrocytes were lysed by treatment with 0.83% NH_4Cl in 0.01 M Tris hydrochloride (pH 7.2). Cell suspensions were washed twice with complete Iscoves modified Dulbecco medium containing 10% low mitogenic fetal bovine serum (AMF, Biological and Diagnostic Products Co., Seguin, Tex.), 40 μg of gentamicin (Carter-Glogau Laboratories, Inc., Glendale, Ariz.) per ml, and 2×10^{-5} M 2-mercaptoethanol.

Brain cyst counts. Brain cysts were counted microscopically in a 50-μl aliquot of brain suspension from infected mice (day 45 p.i.) as described above.

Sabin-Feldman dye test for IgG. The dye test for IgG was performed by using a modification of the method described by Sabin and Feldman (24). Briefly, the serum to be tested was diluted two- or fourfold in phosphate-buffered saline (PBS), pH 7.2, in round-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). A 50-μl suspension of 2×10^6 living tachyzoites per ml in PBS containing 40% human normal serum (as a source of complement) was added to each well. The microtiter trays were shaken for 45 s on a Microshaker II (Dynatech Laboratories) and incubated for 60 min at 37°C. Then, 50 μl of 1% methylene blue in borate buffer, pH 10.8, was added to each well. The trays were allowed to stand at room temperature for 10 min before the test was read.

Agglutination test for IgG. The agglutination test for IgG was performed as described by Desmonts and Remington (7). Briefly, 50 μl of the serum to be tested was diluted two- or fourfold in PBS, pH 7.2, in round-bottomed microtiter trays. To each well, 50 μl of a suspension of Formalin-fixed tachyzoites (4×10^8/ml) was added. The trays were shaken on a Microshaker II for 5 min and allowed to settle at room temperature overnight. Normal mouse serum served as the negative control, and a pool of sera from chronically infected mice served as the positive control.

Enzyme-linked immunosorbent assay for IgM. The enzyme-linked immunosorbent assay was performed as described by Camargo et al. (4). To sensitize plastic surfaces, round-bottomed microtiter plates were filled with *T. gondii* sonic extract containing 20 μg of protein per ml in carbonate-bicarbonate buffer (0.1 M, pH 9.6) and incubated overnight at 4°C. Before use, plates were washed three times with PBS containing 0.05% Tween 20. Then, 0.2 ml of the dilutions of sera to be tested was pipetted into the wells and incubated for 2 h at room temperature. After being washed three times with PBS containing 0.05% Tween 20, the wells were filled with 0.2 ml of rabbit anti-*Toxoplasma* $F(ab')_2$ coupled to alkaline phosphatase. Incubation for 2 h at room temperature was followed by washes; then 0.2 ml of 0.1% disodium *p*-nitrophenyl phosphate in 0.05 M carbonate buffer (pH 9.8) containing 1 mM $MgCl_2$ was added to each well. The reaction was interrupted after 30 min with 1 drop of 2 M NaOH. Serum titers are expressed as the highest serum dilution that is 2 standard deviations above the negative control (normal mouse serum). Pooled sera from infected mice served as a positive control.

Statistics. Statistical analyses were performed by using Student's *t* test or Fisher's exact test.

RESULTS

Survival rate of *T. gondii*-infected immunocompetent and B-cell-deficient mice bearing the *H-2*^{k} haplotype. To determine if B-cell-deficient CBA/N and C3H/He mice, which respond poorly to the B-cell mitogen lipopolysaccharide, are able to survive an acute *T. gondii* infection, these animals and normal CBA/J mice were infected with 20 cysts of the

TABLE 2. *T. gondii*-specific serum IgM levels determined by enzyme-linked immunosorbent assay

Strain	Treatment with sulfadiazine[a]	Log_2 of reciprocal IgM titers ± SD at given day p.i.				
		3	7	14	21	42
CBA/J[b]	−	<4.32	<4.32	6.65 ± 0.058	5.98 ± 1.15	5.65 ± 0.58
	+	<4.32	<4.32	5.32 ± 0	5.32 ± 0	4.65 ± 0.58
CBA/N[c]	−	<4.32	<4.32	<4.32	<4.32	<4.32
	+	<4.32	<4.32	<4.32	<4.32	<4.32
C3H/He[c]	−	<4.32	4.65 ± 0.58	6.32 ± 0	5.99 ± 0.58	6.32 ± 0
	+	<4.32	<4.32	5.65 ± 0.58	5.32 ± 0	5.32 ± 0

[a] Mice were infected in parallel with 20 cysts i.p. Half of the infected mice were treated with sulfadiazine in the drinking water from days 5 to 18 p.i. On indicated days p.i., serum was collected from three mice and individuals were tested for *T. gondii*-specific IgM as described in Materials and Methods. Data represent means of log_2 of reciprocal IgM titers of three individually tested mice.
[b] Differences significant (day 14, $P < 0.05$; days 14 plus 21, $P < 0.05$, by Student's *t* test) between sulfadiazine-treated and untreated mice.
[c] Differences not significant ($P > 0.05$ by Student's *t* test) between drug-treated or untreated mice.

TABLE 3. *T. gondii*-specific IgG antibodies detected by the agglutination test

Strain	Treatment with sulfadiazine[a]	IgG detected in the agglutination test at given day p.i.				
		3	7	14	21	42
CBA/J	−	−	−	+	+	+
	+	−	−	+	+	+
CBA/N	−	−	−	−	+	+
	+	−	−	−	−	+
C3H/He	−	−	−	+	+	+
	+	−	−	+	+	+

[a] Mice were infected in parallel with 20 cysts i.p. Half of the infected mice were treated with sulfadiazine in the drinking water from days 5 to 18 p.i. On indicated days p.i., serum was collected from three mice per group, pooled, and tested for *T. gondii*-specific IgG as described in Materials and Methods.

Me 49 strain; 12 weeks p.i., the number of survivors was determined. Of a total of 31 mice, 14 were treated with sulfadiazine from days 5 to 18 p.i. This drug is known to slow down parasite multiplication, thus allowing the host to develop a more effective immunity. Nearly all of the normal as well as B-cell-deficient mice survived the infection when treated with sulfadiazine from days 5 to 18 p.i. (Table 1). The mortality decreased with sulfadiazine treatment in the CBA/J mice by 22% ($P = 0.119$) and in the CBA/N mice by 39% ($P = 0.018$). Since almost all of the C3H/He mice survived without sulfadiazine administration, a significant influence of the treatment could not be evaluated.

IgM and IgG antibody levels. To determine whether enhanced protection against *T. gondii* correlates with the humoral immune response of the host, infected mice were examined for the presence of parasite-specific antibody during the first 6 weeks of infection. Maximal IgM levels were detectable at 14 days p.i. in CBA/J and C3H/He mice and remained constant or decreased slightly until day 42 p.i. (Table 2); in some of these mice IgM antibody was detectable as early as 7 days p.i. CBA/J mice treated with the drug developed significantly lower IgM levels on day 14 p.i. ($P < 0.05$) but not at any other time tested. None of the CBA/N mice developed detectable IgM antibody during infection regardless of whether they were treated with sulfadiazine.

The agglutination test for IgG was positive only when IgG was detectable by the dye test (Tables 3 and 4). Non-sulfadiazine-treated CBA/J and C3H/He mice developed detectable IgG levels at 2 weeks p.i.; these levels increased until 6 weeks p.i., when the study was concluded. CBA/N mice not treated with sulfadiazine failed to develop detectable IgG levels until 3 weeks p.i., and after 6 weeks, titers still did not reach the levels observed at 2 weeks p.i. in the untreated CBA/J mice (Table 4). Treatment of the infected mice with sulfadiazine decreased serum IgG levels in all three strains of mice tested. Differences between sulfadiazine treated and untreated mice were significant in the CBA/J mice on days 14 and 21 p.i. ($P < 0.001$) and for the CBA/N and C3H/He mice on day 42 p.i. ($P < 0.025$). CBA/N mice developed significantly lower IgG levels than the CBA/J or C3H/He mice whether they were treated with the drug or not ($P < 0.025$).

TABLE 5. *T. gondii*-specific spleen and T-cell proliferation of infected CBA/J, CBA/N, and C3H/He mice

Strain	Treatment with sulfadiazine[a]	Proliferation to *T. gondii* sonic extract ([^{3}H]TdR uptake in cpm)[b]	
		Day 15 p.i. (50 μg/ml)	Day 45 p.i. (10 μg/ml)
CBA/J	−	5,103 ± 751	16,413 ± 2,001
	+	21,805 ± 3,138[c]	6,676 ± 881[c]
CBA/N	−	4,268 ± 621	1,469 ± 209
	+	15,107 ± 2,023[c]	5,218 ± 761
C3H/He	−	2,812 ± 411	2,878 ± 210
	+	13,502 ± 1,500[c]	1,314 ± 285

[a] Mice were infected with 20 cysts i.p. Half of the mice were treated with sulfadiazine in the drinking water from days 5 to 18 p.i. On day 15 or 45 p.i., spleens were harvested.

[b] A total of 4×10^5 spleen cells were cultured in a total volume of 0.2 ml for 5 days. Optimal proliferative responses were induced by 50 μg/ml in spleen cells harvested on day 15 p.i. and by 10 μg/ml in cells harvested on day 45 p.i. During the last 24 h of culture, cells were pulsed with 1.25 μg of [^{3}H]thymidine (TdR), and the uptake of radioactivity by the cells was determined. Data shown are from three pooled spleens, means of triplicate determinations ± SD. Background levels were determined in the same way but in the absence of *T. gondii* sonic extract; they never exceeded 2,205 ± 673 cpm.

[c] Values significantly different ($P < 0.005$ by Student's *t* test) from those of sulfadiazine-untreated mice.

T-cell activation during infection. To investigate a possible correlation between T-cell activity and survival, normal and B-cell-deficient mice infected with *T. gondii* strain Me 49 were tested for antigen-specific spleen cell proliferation in vitro 15 and 45 days p.i. In the absence of drug treatment, none of the three strains tested developed a significant *Toxoplasma*-specific T-cell activation at day 15 p.i. (Table 5). On day 45 p.i., only cells from CBA/J mice proliferated significantly above background levels ($P < 0.005$). When the infected animals were treated with sulfadiazine during the

TABLE 4. *T. gondii*-specific IgG antibody titers determined by the Sabin-Feldman dye test

Strain	Treatment with sulfadiazine[a]	Log_2 of reciprocal IgG titers ± SD at given day p.i.				
		3	7	14	21	42
CBA/J[b]	−	0	0	9.33 ± 0.58	11.0 ± 0	12.0 ± 0
	+	0	0	6.67 ± 0.58	5.33 ± 0.58	10.67 ± 1.16
CBA/N[c]	−	0	0	0	4.67 ± 0.58[d]	10.0 ± 0[e]
	+	0	0	0	0	5.33 ± 2.31[f]
C3H/He[c]	−	0	0	9.33 ± 0.58	11.0 ± 0	11.67 ± 0.58
	+	0	0	9.67 ± 0.58	10.0 ± 0	10.33 ± 0.58

[a] Mice were infected in parallel with 20 cysts i.p. Half of the infected mice were treated with sulfadiazine in drinking water from days 5 to 18 p.i. On indicated days p.i., serum was collected from three mice, and the Sabin-Feldman dye test was performed as described in Materials and Methods. Data represent means of log_2 of reciprocal IgG titers of three individually tested mice.

[b] Differences significant (days 14 and 21, $P < 0.001$ by Student's *t* test) between sulfadiazine-treated and untreated mice.

[c] Differences significant (day 42, $P < 0.025$ by Student's *t* test) between sulfadiazine-treated and untreated mice.

[d] Values significantly different ($P < 0.001$ by Student's *t* test) from those of untreated CBA/J or C3H/He mice.

[e] Values significantly different ($P < 0.05$ by Student's *t* test) from those of untreated CBA/J and C3H/He mice.

[f] Values significantly different ($P < 0.02$ by Student's *t* test) from those of sulfadiazine-treated CBA/J or C3H/He mice.

TABLE 6. Number of brain cysts in *T. gondii*-infected CBA/J, CBA/N, and C3H/He mice

Strain	Treatment with sulfadiazine[a]	No. of mice	Mean brain cyst counts ± SD[b]
CBA/J	−	6	112.0 ± 73.0
	+	7	0.4 ± 0.7
CBA/N	−	7	29.5 ± 10.0[c]
	+	7	0
C3H/He	−	5	25.0 ± 8.0[d]
	+	5	0

[a] Mice were infected with 20 cysts i.p. A part of the infected mice were treated with sulfadiazine in the drinking water from days 5 to 18 p.i. On day 45 p.i., brains of mice were homogenized and the number of brain cysts was determined microscopically.

[b] Differences significant ($P < 0.001$ by Student's t test) between sulfadiazine-treated and untreated mice in all three strains.

[c] Values significantly different ($P < 0.01$ by Student's t test) from those of sulfadiazine-untreated CBA/J mice.

[d] Values significantly different ($P < 0.025$ by Student's t test) from those of sulfadiazine-untreated CBA/J mice.

acute stage of infection, all three strains developed a significant T-cell activation by day 15 p.i. ($P < 0.005$). In contrast, on day 45 p.i. (27 days after termination of the drug treatment), proliferation of T cells from all three strains tested dropped to background levels.

Brain cyst counts. Brain cyst counts were compared in normal and B-cell-deficient mice. Treatment of normal and B-cell-deficient mice with sulfadiazine resulted in a suppression of cyst formation (Table 6). No cysts were detected in brains of C3H/He and CBA/N mice following sulfadiazine treatment; treated CBA/J mice had a mean of only 0.4 cyst per 50 μl of brain suspension. Mice not treated with the drug developed significantly more cysts ($P < 0.002$) in comparison to sulfadiazine-treated mice. Interestingly, normal CBA/J mice not treated with the drug developed significantly ($P < 0.01$ to $P < 0.025$) greater amounts of cysts than B-cell-deficient CBA/N or C3H/He mice (Table 6).

DISCUSSION

Cell-mediated immunity plays a major part in development of protection against many parasitic infections, especially against reinfection (6). In toxoplasmosis, T-cell-mediated immunity also seems to play an important role (20), and in H-2^k mice it seems to be influenced by regulatory B cells (3). To further elucidate the role of humoral and cellular immunity in mediating protection, normal CBA/J, C3H/He, and B-cell-deficient CBA/N mice were compared for their ability to mount an immune response against an acute *T. gondii* infection.

CBA/N mice are known to have an X-linked defect leading to low serum IgM levels, an inability to respond to certain thymus-independent antigens, and low responses to thymus-dependent antigens (2, 27, 28). C3H/He mice are known to have low leukocyte counts. They fail to respond strongly to lipopolysaccharide, which might be related to a qualitative or a quantitative lack of proper B-cell function (1). All three mouse strains used were of the *H-2*^{k} haplotype and were therefore comparable except for their B-cell compartment.

Our results show that the survival of mice following an acute *Toxoplasma* infection does not correlate with enhanced serum IgG or IgM levels. On the other hand, antibody seems to facilitate development of resistance as evidenced by increased mortality of CBA/N mice in comparison to CBA/J and C3H/He mice. These latter results are similar to those reported by Lindberg and Frenkel in nude mice (22).

To optimize the protective immune response, animals were treated with sulfadiazine during the acute stage of infection (days 5 to 18 p.i.). Sulfadiazine is a competitive antagonist of *p*-aminobenzoic acid and thus prevents normal synthesis of folic acid by parasites. It does not affect mammalian cells, because they require preformed folic acid and cannot synthesize it (13). Therefore, it is routinely used to convert lethal *Toxoplasma* infection to lifelong chronic infections by inhibiting the parasite's multiplication, which enables the host to respond more effectively to the parasite. Drug treatment strengthened the protective immune mechanism in all three mouse strains tested. However, it did not favor antibody production, because CBA/J and CBA/N mice developed a diminished humoral response as compared to animals not treated with the drug. In addition, drug-treated CBA/N mice produced almost no antibody, but they survived the acute infection as well as CBA/J or C3H/He mice. Sulfadiazine treatment may thus inhibit production of ineffective, immune suppressive, or autoreactive antibody. Such a hypothesis would be consistent with observations in other infection and cancer models. For example, Jerusalem et al. (15) described an increased mortality in mice infected with *Plasmodium berghei* that was caused by passive as well as active immunization against the parasite. Casey et al. (5) and Kalisa and Snell (17) reported that immunization of mice with soluble tumor antigen often leads to enhanced tumor growth. High IgG titers induced by these immunizations correlated with reduced protection of the host. These observed effects described as "immunologic enhancement" might be due to masking of parasite or tumor antigens by antibodies or to enhanced autoimmunity. In addition, it is possible that in our infection model treatment with sulfadiazine favors the production of other antibody subclasses, which might be more effective in the development of a protective response. Our results demonstrate that drug treatment reduced mortality and brain cyst formation and enhanced *Toxoplasma*-specific T-cell activation during the early stage of infection (day 15 p.i.) in all three mouse strains tested. In contrast, without drug treatment only the CBA/J mice showed delayed development of antigen-reactive T cells. Therefore, early activation of the T-cell compartment seems to be more efficient in inducing protection against the acute infection. The cellular immunity of the drug-treated CBA/N mice, including nonspecific killing, might become so effective that they are able to overcome their deficiency in B-cell function and are thus able to develop a resistance comparable to that of the sulfadiazine-treated CBA/J mice. The surprising contradiction that sulfadiazine treatment seems to decrease the antigen-specific T-cell response in normal CBA/J and C3H/He mice on day 45 p.i. but seems to increase it in the CBA/N mice might be related to differences between the mouse strains in the degree of B-cell-mediated L3T4$^+$ T-cell suppression. In addition, blocking of interleukin-2 by an interleukin-2 inhibitor recently described by us in this model (3) and by others in mitogen-induced interleukin-2 production (14) might be responsible for the observations reported here.

Brain cyst formation is a well-known feature in toxoplasmosis. However, it is not yet known how brain cyst formation is related to protective immune mechanisms since both immune and nonimmune mechanisms have been implicated in brain cyst formation (20). From our results, it can be concluded that lower brain cyst counts reflect a better protection against the parasite in normal CBA/J and C3H/He mice.

In B-cell-deficient CBA/N mice, the lack of high IgG and

IgM levels leads to significantly less brain cyst formation as compared to the normal CBA/J mice. This observation suggests that high humoral responses do not inhibit, but more likely favor, cyst formation by mechanisms discussed above. The extremely low antibody titers in the CBA/N mice, in addition to our previous findings, implicate that a major part of protective immunity against *T. gondii* is cell mediated and that cell-mediated immunity also controls brain cyst formation.

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